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ctDNA whole exome sequencing in pancreatic ductal adenocarcinoma unveils organ-dependent metastatic mechanisms and identifies actionable alterations in fast progressing patients

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ABSTRACT

Understanding progression mechanisms and developing new targeted therapies is imperative in pancreatic ductal adenocarcinoma (PDAC). In this study, 80 metastatic PDAC patients were prospectively recruited and divided into discovery (n=37) and validation (n=43) cohorts. Tumor and plasma samples taken at diagnosis were pair analyzed using whole exome sequencing (WES) in patients belonging to the discovery cohort alone. The variant allele frequency (VAF) of KRAS mutations was measured by ddPCR in plasma at baseline and response assessment in all patients. Plasma WES identified at least one pathogenic variant across the cohort, uncovering oncogenic mechanisms, DNA repair, microsatellite instability, and alterations in the TGFb pathway. Interestingly, actionable mutations were mostly found in plasma rather than tissue. Patients with shorter survival showed enrichment in cellular organization regulatory pathways. Through WES we could identify a specific molecular profile of patients with liver metastasis, which exhibited exclusive mutations in genes related to the adaptive immune response pathway, highlighting the importance of the immune system in liver metastasis development. Moreover, KRAS mutations in plasma (both at diagnosis and persistent at follow-up) correlated with shorter progression free survival (PFS). Patients presenting a reduction of over 84.75 % in KRAS VAF at response assessment had similar PFS to KRAS-negative patients. Overall, plasma WES reveals molecular profiles indicative of rapid progression, potentially actionable targets, and associations between adaptive immune response pathway alterations and liver tropism.

Abbreviations: cfDNA, cell free DNA; CNVs, copy number variations; ctDNA, cell free tumor DNA; ddPCR, droplet digital protein chain reaction; FFPE, Formalin-Fixed Paraffin-Embedded; HCUV, Hospital Clínico Universitario de Valencia; mPDAC, metastatic pancreatic ductal adenocarcinoma; OS, overall survival; PDAC, pancreatic ductal adenocarcinoma; PFS, progression free survival; SNVs, single nucleotide variants; TT, tumor tissue; UMI, unique molecular identifier; VAF, variant allele frequency; WBC, white blood cells; WES, whole exome sequencing.

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Brief Commentary

Background: Molecular characterization of pancreatic cancer is challenging due to the abundance of stromal and inflammatory cells in paraffin-embedded samples. Whole exome sequencing (WES) in plasma overcomes the limitations of biopsy, potentially detecting targetable alterations and enabling tailored approaches.

Translational Significance: Our plasma WES analysis distinguishes slow and fast progressing patients under standard treatment, emphasizing the role of the adaptive response pathway in liver metastasis. Additionally, ctDNA WES improves actionable alteration identification, enabling treatment in personalized clinical trials. Setting a *KRAS* variation cutoff aids in identifying responders and avoiding ineffective treatments. This non-invasive approach can transform our understanding of pancreatic cancer, refining diagnostic precision and therapeutic strategies.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) presents a poor prognosis, exhibiting one of the lowest survival rates among solid tumors.[1] With mortality rates almost equaling new cases annually and increasing incidence and mortality, it ranks the third leading cause of cancer death in most developed countries.[2,3] Chemotherapeutic agents are the only approved drugs for PDAC treatment, and are administered based on clinical factors rather than biomarker-guided, thus having a palliative function in advanced disease. These non-targeted treatments achieve a median survival of less than a year, underscoring the primary factor contributing to the dismal prognosis observed in these patients.[4-6] The only exception is Olaparib, used in the rare event of germline BRCA mutations, demonstrating a median overall survival (OS) of approximately 19 months.[7]

Multi-platform analysis has uncovered a complex molecular landscape in PDAC, affirming distinct molecular subtypes and indicating potential targetable biomarkers.[8] While there are no prospective randomized studies supporting the benefits of personalized therapy in PDAC, retrospective analyses of real-world data suggest that targeted therapies may enhance both progression-free survival (PFS) and OS. Moreover, several early phase trials are recruiting PDAC patients to evaluate the potential role of a molecular matching approach with *KRAS G12C/D, PIK3CA* or *FGFR* inhibitors.[9,10] Notably, emerging tyrosine kinase inhibitors such as sotorasib and adagrasib are designed to selectively target the p.G12C mutation in *KRAS*, along with MTRX1133 for p.G12D, thereby defining a promising avenue for targeted therapy in this prevalent subgroup of *KRAS*-mutated PDAC patients with a poorer prognosis.[11-13]

A potential solution to the challenge of obtaining an optimal molecular landscape in precision medicine from tumor tissue (TT) is the use of cell-free tumor DNA (ctDNA) for molecular characterization, as observed in other tumors.[14-17] Evidence suggests that ctDNA may hold prognostic value in various tumor types.[18-23] Despite these potential applications, ctDNA is not yet standardized or approved for clinical decision-making. The detection of *KRAS* mutations, which are nearly universal in PDAC,[24] using precise techniques such as droplet digital PCR (ddPCR), demonstrates prognostic value[25-28] and the ability to anticipate treatment response.[22,29,30]

The aim of this study is to overcome the limitations of TT analysis and open new avenues to a precision approach for metastatic PDAC (mPDAC), by discovering potential targetable mutations in plasma that are undetectable in tumor tissue (TT). Identifying a specific molecular profile via WES could help stratify the population by prognosis and pinpoint those who could potentially develop liver metastases. Additionally, evaluating the dynamic changes in the variant allele frequency (VAF) of *KRAS* mutations by ddPCR in ctDNA between baseline and the time of first tumor assessment could help classify prognosis across advanced PDCA patients.

Material and methods

Patient cohort, study design and clinical samples

A total of 80 eligible mPDAC patients at Hospital Clínico Universitario de Valencia (HCUV), Spain were prospectively recruited for first-line chemotherapy between June 2016 and August 2022. PDAC diagnosis was pathologically confirmed, and treatment adhered to clinical practice guidelines. Ethical approval (Protocol Number: 2017.118) was obtained from HCUV Ethics Committee, and the study was aligned with good clinical practice, national laws, and the Declaration of Helsinki. All patients provided written informed consent.

TT was obtained pre-treatment, and blood samples were collected at baseline and every 2–3 months (follow-up samples), coinciding with computed tomography scan assessments. Monitoring continued until patient demise, inability to reach the hospital, refusal, or loss to follow-up.

In the discovery cohort (June 2016–April 2020, 37 patients), WES was conducted on baseline blood and TT samples, and *KRAS* mutations were analyzed using ddPCR on plasma samples at baseline and follow-up visits. In a separate validation cohort (May 2020–August 2022, 43 cases), *KRAS* mutations in ctDNA were assessed using ddPCR at baseline and initial tumor response evaluation. Supplementary Fig. S1 presents the CONSORT diagram (Fig. S1A) and the study design illustration (Fig. S1B).

DNA extraction and whole exome library preparation

The formalin-fixed paraffin-embedded (FFPE) tissue block was macro-dissected, and specific kits were employed for DNA extraction: the AllPrep DNA/RNA FFPE kit (Qiagen) for tumor DNA from FFPE sections, the Chemagic DNA blood kit (Chemagen) for germline DNA from matched white blood cells (WBC), and the QIAamp Circulating Nucleic Acid kit (QIAGEN) for cfDNA from 4 mL plasma samples. All procedures followed the manufacturer's instructions. DNA from tumor and WBC was quantified using the QuantiFluor dsDNA System (Promega). Assessment of cfDNA quantity and quality was conducted using the Cell-free DNA ScreenTape Assay (Agilent). cfDNA samples falling below the 70 % threshold were considered unacceptable.

Libraries were prepared with 100 ng tumor, 100 ng WBC, and 10-40 ng cfDNA inputs. KAPA HyperPlus kit (Roche) and IDT UDI-UMI indexes were used for tumor and germline DNA. Library preparation followed KAPA HyperCap Workflow v3, with a modification using 5 mL of 15 mM UDI-UMI solution in ligation. Illumina Primer Mix was used for pre-capture PCR. For cfDNA, KAPA HyperPrep kit (Roche) with the same adapters was used, with adjusted adapter dilution, employing an 11-cycle pre-capture PCR, and post-PCR purifications with 50 mL KAPA HyperPure Beads. Elution occurred in 50 mL of 10 mM Tris-HCl (pH 8.0), followed by another bead incubation. Pre-capture libraries were combined, and exome enrichment used KAPA HyperExome kit (Roche) following the manufacturer's instructions, with the same post-PCR purification for cfDNA exome samples.

Quality control and sequencing

Pre- and post-capture library quality was assessed using the HS D1000 ScreenTape Assay (Agilent) with a 20-fold dilution. Sequencing utilized either the HiSeq 3000 (Illumina) or NovaSeq 6000 (Illumina) platforms with a paired-end (PE) 150-base pair strategy, including an extended i7 read of 17 cycles for Unique Molecular Identifier (UMI) reading.

Sample coverage was assessed at varying depths: 50x for WBC, 100x for FFPE, and 250x for cfDNA. Samples achieving or exceeding 75 % of

Table 1
Patient characteristics.

Characteristics		Discovery cohort n=37 (%)	Validation cohort n=43 (%)	p-value	All patients n=80 (%)
Age (years)	Median (range)	66.0 (57- 69)	67 (57 - 72.5)	0.372	66 (57-71)
Gender	Male	14 (37.8)	23 (53.4)	0.184	37 (46.3)
	Female	23 (62.2)	20 (46.5)		43 (53.7)
ECOG	0	3 (8.1)	2 (4.7)	0.731	5 (6.2)
Performance status	1	24 (64.9)	31 (72.1)		55 (68.8)
	2	8 (21.6)	9 (20.9)		17 (21.3)
	3	2 (5.4)	1 (2.3)		3 (3.7)
CA 19.9 at baseline	≤ 27 U/mL* (low)	3 (8.1)	6 (14)	0.126	9 (11.2)
	>27 U/mL (high)	34 (91.9)	37 (86)		71 (88.8)
Metastatic sites	Liver	25 (67.6)	29 (67.4)	1	54 (67.5)
	Peritoneum	11 (29.7)	13 (30.2)	1	11 (13.8)
	Lung	10 (27)	7 (16.3)	0.281	7 (8.8)
	Other	3 (8.1)	8 (18.6)	0.208	1 (1.2)
Number of metastatic sites	1	28 (75.7)	30 (69.8)	0.461	58 (72.5)
	2	5 (13.5)	10 (23.3)		15 (18.8)
	More than 2	4 (10.8)	3 (7)		7 (8.8)
First line of treatment	None	1 (2.7)	0 (0)	0.174	1 (1.2)
	Gemcitabine	7 (18.9)	6 (14)		13 (16.3)
	Gemcitabine + Nab-paclitaxel	25 (67.6)	24 (55.8)		49 (61.3)
	FOLFIRINOX	4 (10.8)	12 (27.9)		16 (20)
	Other	0 (0)	1 (2.3)		1 (1.2)
Overall survival in days** (range)		249 (27-NR)	263 (9-NR)	0.460	245 (9-NR)

* 27 U/mL is our upper normal limit for CA 19.9
** One patient in the discovery cohort and ten patients in the validation cohort were alive at the time of the analysis, so the upper limit of survival has not been reached (NR).

the target depth were deemed suitable. Median coverage values were 139.5X for WBCs, 102.53X for FFPE, and 425.43X for plasma samples.

Fastq Preprocessing and read mapping

Raw sample quality control was performed using FastQC (v0.11.8), followed by adapter trimming (Cutadapt v2.10), and reads below Q30 mean quality were removed in FASTQ preprocessing using PrinSeq (v0.20.4). Sequencing reads were aligned to the hg38 human reference genome using BWA (v0.7.17). BAM files underwent post-processing following PICARD (v2.18.6) and GATK (v4.2.0.0) best practices. UMI extraction and deduplication were performed using Umi-tools (v1.0.1).

Variant calling and somatic variant prioritization

Variant calling was carried out with Mutect2 (GATK v4.2.0.0) and Lofreq (v2.1.5) for TT, plasma, and WBCs, with HaplotypeCaller (GATK, Sarek pipeline v2.7.1) for germline variants. VAF thresholds: 5 % for TT, 0.1 % for plasma. Mutations were considered true if they had at least 3 alternative alleles to ensure the quality and confidence of the alteration. Merged variants from both callers underwent WBC extraction and VEP annotation (Ensembl v102). Clonal hematopoiesis of indeterminate potential variants in plasma were removed. Oncogenic somatic variants were annotated using COSMIC (v94), OncoKB API (v1), and an in-house database, with manual curation. Variant prioritization followed OncoKB levels. Copy number variations (CNVs) in FFPE samples were detected using CNVkit 0.9.7, VarScan 2.4.4, and CNV facets 0.16.0, while CONTRA 2.0.8 and WiseCondorX 1.2.4 handled liquid biopsies.

The HD780 (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0) and HD816 standards (preserved in synthetic plasma and extracted with the QIAamp® Circulating Nucleic Acid kit), both from Horizon Discovery, were used as circulating DNA controls. Both samples contain variations in the *KRAS, NRAS, PIK3CA*, and EGFR genes, including insertions and deletions, at 0.0 % (wt), 0.1 %, 1.0 %, and 5.0 % for samples HD734 and HD827. Specificity was 100 % for both control samples, while sensitivity was 100 % for the 1.0 % and 5.0 % variants, falling to 87.5 % for variants within the 0.1 % range.

ctDNA ddPCR analysis

ddPCR was performed on a QX200 ddPCR system (Bio-Rad) using TaqMan chemistry, following the manufacturer's instructions. A mixture was prepared containing 20 ng of cfDNA in 8 μL, 10 μL of supermix without dUTP (Bio-Rad), and 2 mL of FAM/HEX probes targeting the specific genetic changes. Emulsions were analyzed with default settings for rare event detection and appropriate fluorophore identification. The mutations in *KRAS* codons G12 and G13 were determined using probes from the "ddPCR™ *KRAS* G12/G13 Screening Kit #1863506."

Statistical analysis

To identify genes that exhibited exclusive or significant mutations between different patient groups, we performed a gene-by-gene odds ratio analysis. The significance of each odds ratio was assessed using Fisher's exact test. To shed light on the functional implications of genes exclusively mutated in patients with poor prognosis (less than 11 months) and those with liver tropism compared to "other" metastases, we conducted Gene Set Enrichment Analysis (GSEA) and examined Gene Ontology Biological Pathways (GOBP)

Continuous demographic, clinical and laboratory variables were analyzed for normality using the Shapiro-Wilk test. Normally distributed data were reported as mean (SD) and non-normally distributed data as median (IQR). Cohort comparisons used t-tests or Kruskal-Wallis tests for normally and non-normally distributed data, respectively. Categorical data were reported as n (%) and analyzed by Chi-squared ($\chi 2$) test or Fisher's exact test if cell count was lower than 5. Cox regression was used to analyze survival data, and the Log-Rank test to compare Kaplan-Meier curves. P-value adjustment according to Benjamini-Hochberg's[31] method was used to adjust for multiplicity.

The discovery cohort was used to calculate the optimal cut-off for *KRAS* VAF reduction using maximally selected rank statistics[32] or time to disease progression. This value was then included in a multivariable Cox model with forward-backward stepwise variable selection based on the Akaike Information Criterion[33] considering age (<65 vs. ≥65 years old), ECOG status (0, 1 or ≥2), presence of liver metastasis (yes vs. no) and CA 19.9 level (above vs. below the upper limit of normality). Proportional hazard assumption was tested for the final model.

Analyses were conducted in R (version 4.0.2)[34] with a significance cutoff of 0.05.

Results

Patient characteristics

A total of 80 untreated patients diagnosed with mPDAC, comprising 37 in the discovery cohort and 43 in the validation cohort, were enrolled in this study. The median age of the patients was 66 years. The majority (68.8 %) had an ECOG performance status of one at baseline, and 67.5 % of patients presented with liver metastasis at the time of diagnosis. The primary first-line chemotherapy regimen administered in most cases was Gemcitabine plus Nab-paclitaxel (61.3 %). The median duration of follow-up was 245 days. Key clinical characteristics of patients are summarized in Table 1. In the discovery cohort, ctDNA WES was available for 29 of the 37 patients (78.4 %).

Plasma-based WES elucidates the molecular landscape of metastatic PDAC

To characterize the molecular landscape of pancreatic cancer, we performed WES in 10 TT, 29 plasma and matched WBCs of patients with mPDAC belonging to the discovery cohort. After filtering out germline variants by comparison with WBCs, we prioritized single nucleotide variants (SNVs) and small insertions and deletions (INDELs) in tissue samples. We conducted a targeted study in a 30 human gene set to identify pathogenic mutations with the greatest influence on the development and evolution of pancreatic tumors.[35]

The analysis revealed at least one pathogenic variant across the cohort, with *TP53* (79 %), *KRAS* (55 %), and *BRCA2* (28 %) emerging as the most prevalent mutated genes. Mutations were categorized and the association with specific cancer pathways was investigated. Finally, four types of enriched pathways were highlighted, belonging to oncogenic mechanisms, DNA repair, microsatellite instability, and the TGF pathway [Fig. 1A].[36]

CNV analysis showed alterations in 15 out of 29 patients (51.7 %). Specifically, we observed *MSH2* amplification in 5 patients (17 %) [Supplementary Fig. S2]. Conversely, *TP53* losses were detected in four

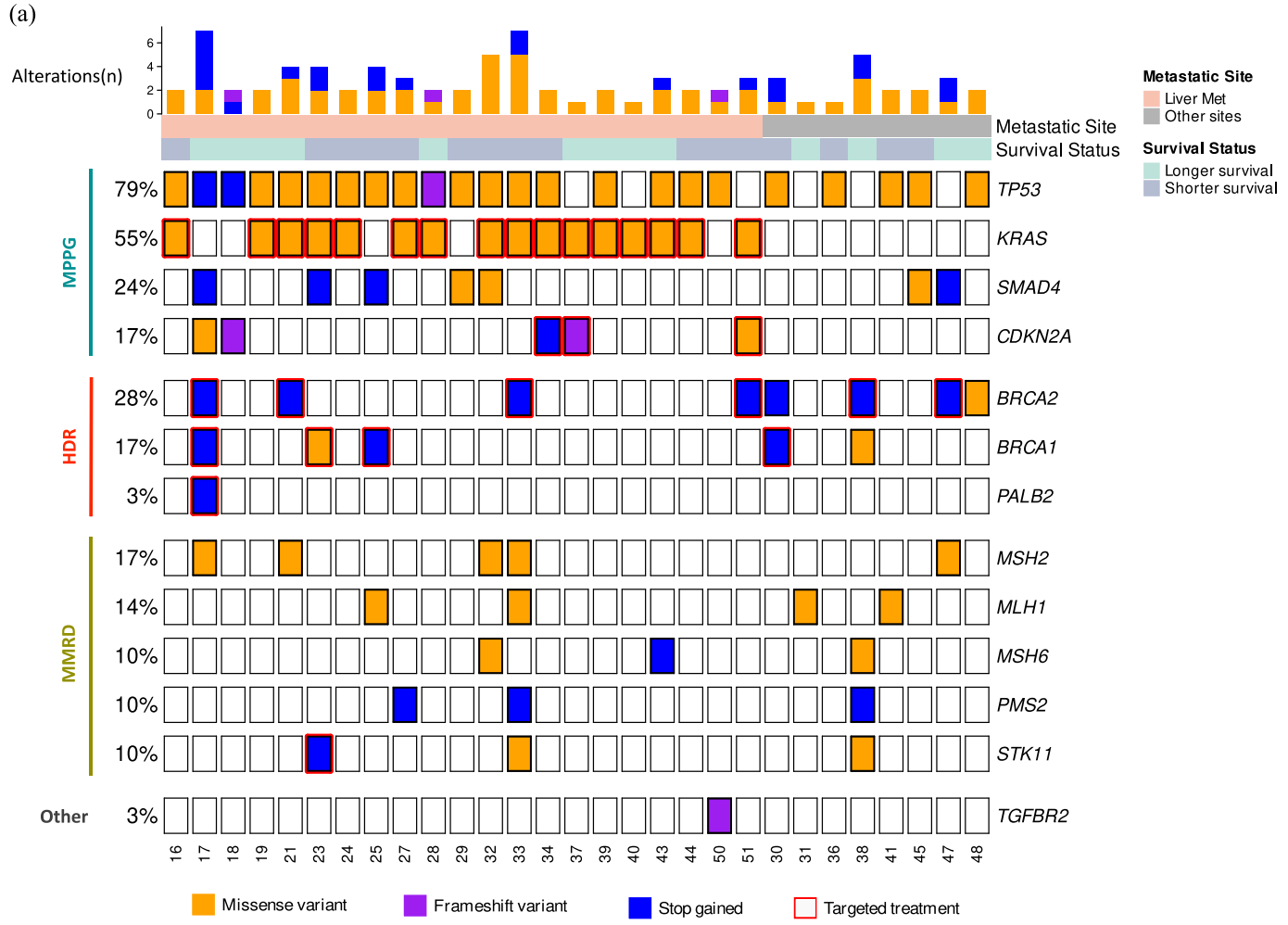

Fig. 1. Plasma-based WES unveils the molecular landscape of metastatic PDAC and enhances actionable target mutation identification. A. Molecular landscape of SNVs identified in plasma samples from 29 patients with advanced pancreatic cancer. Each box represents a patient-specific mutated gene, with color indicating mutation type. The Y-axis in the upper portion of the figure displays the point mutation count in each patient. In the top bars, below that axis, pink identifies patients with hepatic metastases, and grey those with metastases in other locations. In green, patients with a survival of more than 11 months are represented, and in grey, those with a shorter survival. The genes are arranged from top to bottom, featuring the most prevalent pancreatic cancer-associated genes (MPPG), genes related to homologous recombination deficiency (HDR), mismatch repair deficiency (MMRD), and TGFBR2. 'Targeted treatment' denotes potentially actionable treatments as per OncoKB. B. Molecular landscape of SNVs detected in paired tissue and plasma samples. Comparative molecular landscape of oncogenic mutations in paired baseline samples from 8 pancreatic cancer patients, comprising tissue and plasma. Each box represents a mutated gene of a specific patient, with color denoting its presence in plasma (blue), tumor tissue (purple), or both (green). The Y-axis displays the mutation count in each patient.

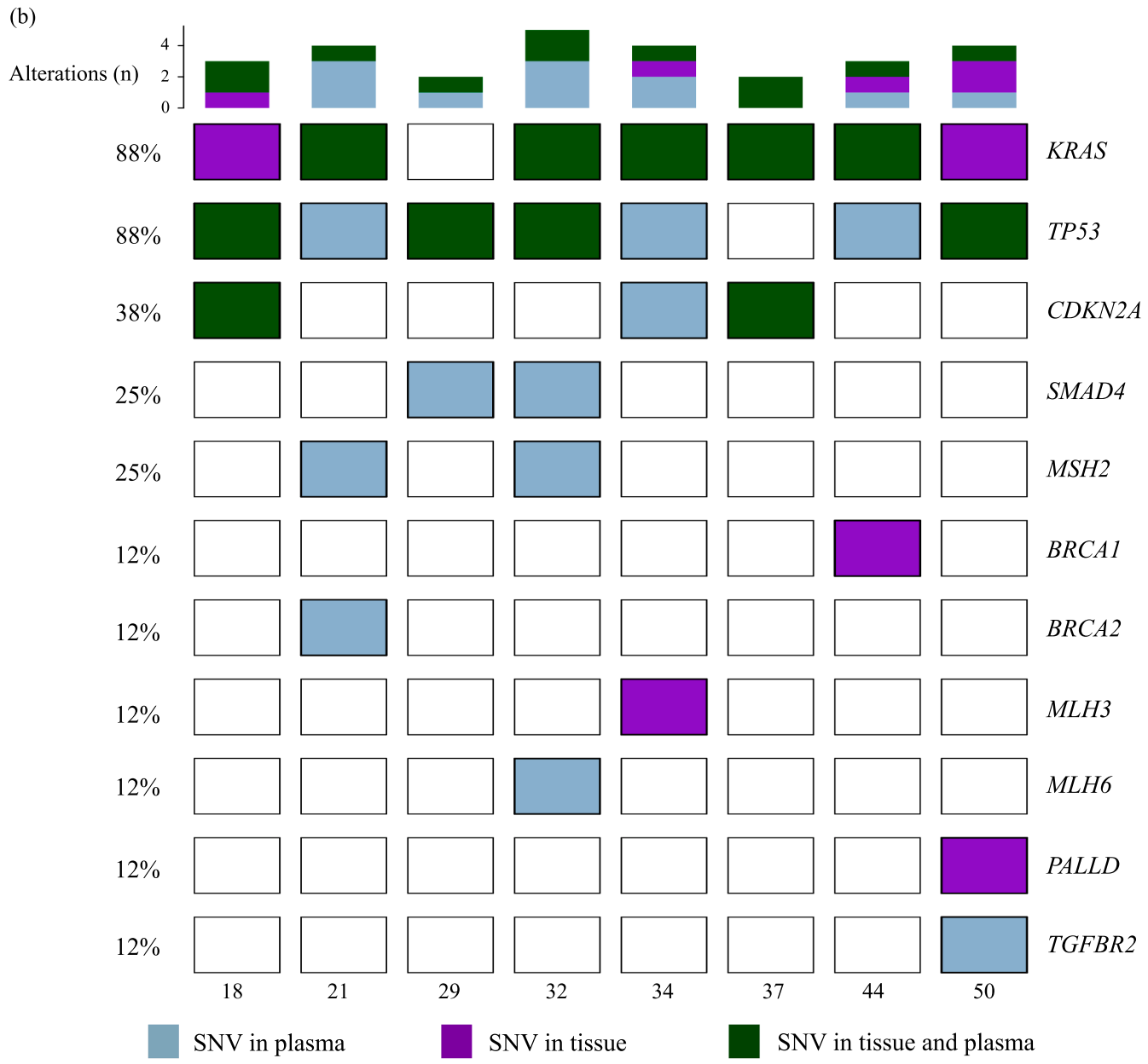

Fig. 1. (*continued*).

patients (14 %), while amplification was seen in one patient (3 %). Interestingly, *MLH1* was altered in 14 % of the cohort (4/29 patients).

In addition, WES analysis of WBCs showed pathogenic germline variants in 10.3 % (3/29) of patients (Supplementary Table S1). These variants included mutations in *TP53, RECQL4* and *AXIN1*, highlighting the potential role of germline variants in PDAC development.

Plasma-based WES enhances the ability to identify actionable target mutations

To explore targeting genetic alterations in plasma due to intratumoral heterogeneity, 8 paired samples of TT and plasma were analyzed. Individual patient-level comparison revealed roughly 20 % concordance regarding shared alterations in plasma and TT (Supplementary Fig. S3A). Notably, concordant mutations showed a significantly higher variant VAF than plasma-exclusive variants (t-test; p-value = 0.007; concordant median VAF: 7.423 %; plasma-exclusive median VAF: 1.387 %); 50 % of the plasma-exclusive variants were found in the VAF range of 0.1–1 %.

Interestingly, *KRAS* mutations were observed in both plasma and TT in 5 patients (62.5 %), while *TP53* mutations were detected in 4 patients (50 %) (Supplementary Fig. S3B). In particular, *KRAS* mutations were found exclusively in TT in 2 patients and confirmed in plasma only via ddPCR (0.6 % in patient 18 and 2.08 % in patient 50). In contrast, *TP53* mutations were identified only in plasma in three patients. These findings suggest that although *TP53* and *KRAS* mutations are common in the primary tumor, *TP53* mutations exhibit higher subclonality within our cohort. Beyond these classical molecular alterations, nevertheless, most other mutations were detected in plasma rather than TT, as expected (Fig. 1B).

Exploring a potential molecular-matched approach derived from this study, we identified seven druggable mutations in tissue samples and 11 in plasma (such as *PTEN, BRCA2*, and *TSC2*), showcasing a broader spectrum of targetable alterations in plasma which could expand therapeutic options for patients. However, focusing solely on TT alteration, we identified targetable mutations in genes like *MTOR, EZH2*, and

Table 2
Potential targetable mutations detected in plasma based on the OncoKb database.

Gene	HPAC *	Tissue	Druggable mutations (n)	Patients (%)	VAF (median)	Consequences	Treatment
KRAS^{G12D}	Yes	Yes	8	28 %	38 %	Missense variant	MTRX1133
KRAS^{G12C}	Yes	No	1	3 %	4 %	Missense variant	Sotorasib/Adagrasib
NF1	No	No	6	21 %	1.8 %	Stop gained	Trametinib/Cobimetinib/Selumetinib
BRCA2	Yes	No	6	21 %	1.7 %	Stop gained	Rucaparib/Talazoparib/Olaparib/Niraparib
TSC2	No	No	5	17 %	0.5 %	Missense variant Stop gained	ABI-009/Everolimus
BRCA1	Yes	Yes	4	14 %	0.8 %	Stop gained Missense variant	Rucaparib/Talazoparib/Olaparib/Niraparib
ARID1A	No	Yes	4	14 %	4.7 %	Frameshift variant Stop gained	Tazemetostat/PLX2853
ATM	No	No	3	10 %	1.6 %	Stop gained	Olaparib
CDKN2A	Yes	Yes	3	10 %	5.6 %	Stop gained Missense variant	Abemaciblib/Ribociblib/Palbociclib
PTEN	No	No	3	10 %	1.1 %	Missense variant	AZD8186/GSK2636771
ERBB2	No	No	2	7 %	0.8 %	Missense variant	Trastuzumab-Deruxtecan/Neratinib/Trastuzumab-entansine
RET	No	No	2	7 %	0.9 %	Missense variant	Pralsetinib/Selpecatinib
CHEK2	No	No	2	7 %	1.9 %	Splice donor variant	Olaparib
PDGFRA	No	No	1	3 %	0.8 %	Missense variant	Sunitinib/Regorafenib/Avapritinib/Ripretinib/Imatinib
ERCC2	No	No	1	3 %	0.7 %	Splice donor variant	Cisplatin
STK11	Yes	No	1	3 %	0.5 %	Stop gained	Bemcintinib
ZRSR2	No	No	1	3 %	1.6 %	Stop gained	H3B-8800
CDK12	No	No	1	3 %	0.7 %	Splice donor variant	Pembrolizumab/Olaparib/Cemiplimab/Nivolumab
NRAS	No	No	1	3 %	1.0 %	Missense variant	Trametinib/Binimetinib/Panitumumab /Selumetinib/ Cetuximab/ Cobimetinib
FANCL	No	No	1	3 %	1.4 %	Splice donor variant	Olaparib
BARD1	No	No	1	3 %	1.0 %	Splice acceptor variant	Olaparib
RAD51D	No	No	1	3 %	0.4 %	Stop gained	Olaparib
FGFR1	No	No	1	3 %	0.8 %	Missense variant	Erdafitinib/Infigratinib/Debio1347/AZD4547
ALK	No	No	1	3 %	0.9 %	Missense variant	Lorlatinib
PALB2	Yes	No	1	3 %	0.6 %	Stop gained	Olaparib

* HPAC: hallmark pancreatic adenocarcinoma. Only the two potentially actionable mutations harbored by the *KRAS* gene are shown.

SF3B1 in 10 % of cases (Table 2).

As predicted, WES analysis surpassed the PDAC panel[35] in detecting targetable mutations, showing 70 druggable mutations across 24 genes compared to 33 mutations in six genes. Notably, the PDAC panel detected mutations in 72 % of patients, while WES analysis identified mutations in 93 % of patients, showcasing its superior capacity for identifying somatic variants amenable to targeted therapy and opening up new treatment possibilities.

ctDNA WES identifies fast progressing patients and liver tropism

Via analysis of molecular alterations in plasma we were able stratify our cohort into two different groups according to prognosis. Specifically, individuals with a poorer prognosis (survival <11 months) were identified, aligning with the median OS obtained in phase III trials of presently recommended first-line treatments[4-6,37]), demonstrating somatic mutations in 32 genes exclusively identified in baseline plasma. Notably, these genes included oncogenes such as *MTOR, CDK12*, and *EPHA7* (Fig. 2A). Through functional enrichment analysis using Gene Ontology (GO), we noted that the mutated genes within this patient subgroup are linked to pathways involving cellular organization, cellular movement, and apoptotic protection. In contrast, patients with longer survival showed mutations only in DNA regulation and repair genes such as nine *ZNF* genes or in *MLH3*. Exploring oncogenic mutations in non-traditional pancreatic cancer genes can provide valuable insight into aberrant signaling pathways. For instance, the *CDK12* mutations present in patients with poor prognosis emerge as a potential therapeutic target.[38]

Noteworthy findings were observed regarding baseline tropism for organ-specific metastases in pancreatic cancer patients. A total of 25 out of 37 patients developed liver metastases. These patients presented somatic mutations in *KRAS, LAMA1, FGFR1*, or *IFFO1*, while those with other metastatic sites were wild-type for those genes (Fig. 2B). Patients with liver metastases also exhibited enrichment in GO terms related to mutations in adaptive immune response, including genes like *HLA-H, HLA-DRB1*, or *TRBV6-7*, indicating a potential role for the immune system in hepatic disease development. In conclusion, both tumor features (such as the *KRAS* mutation) and this immune profile could be responsible for the development of liver metastasis.

In total, 31 % of patients exhibited targetable mutations in the *KRAS* gene in plasma samples, highlighting the importance of detecting targetable mutations in plasma before liver metastasis development. The predominant mutation was the G12D variant, observed in both plasma (28 % of patients, detailed in Table 2) and tissue (50 % of patients; Supplementary Table S2). In contrast, G12C alterations were exclusively identified in plasma (present in 3 % of cases).

This evidence could support a change in the current treatment scenario, with the introduction of specific *KRAS* G12C/D inhibitors to prevent liver metastasis development in patients sharing the described molecular features.

Prognostic value of monitoring and detecting KRAS in ctDNA

To assess the prognostic value of plasma-detected *KRAS* G12/G13 mutations using ddPCR in the discovery cohort, we evaluated PFS and OS based on the presence of detectable *KRAS* mutations at baseline (n=37) and at first response assessment (n=24). At baseline, 27 patients (73 %) had detectable plasma *KRAS* mutations. At time of the first response evaluation, eight patients (33.3 %) maintained *KRAS* detectable in plasma (as shown in Supplementary Table S3 and Supplementary Fig. S4).

Patients with *KRAS* mutations at both baseline and first tumor assessment had worse PFS than those with undetectable mutations in plasma (HR= 2.315, CI 95 %= 1.027-5.217, p= 0.038 and HR= 2.838, CI 95 %= 1.104-7.296, p= 0.024 respectively, Supplementary Fig. S5 A–B). Furthermore, *KRAS* mutation detection in plasma at first tumor

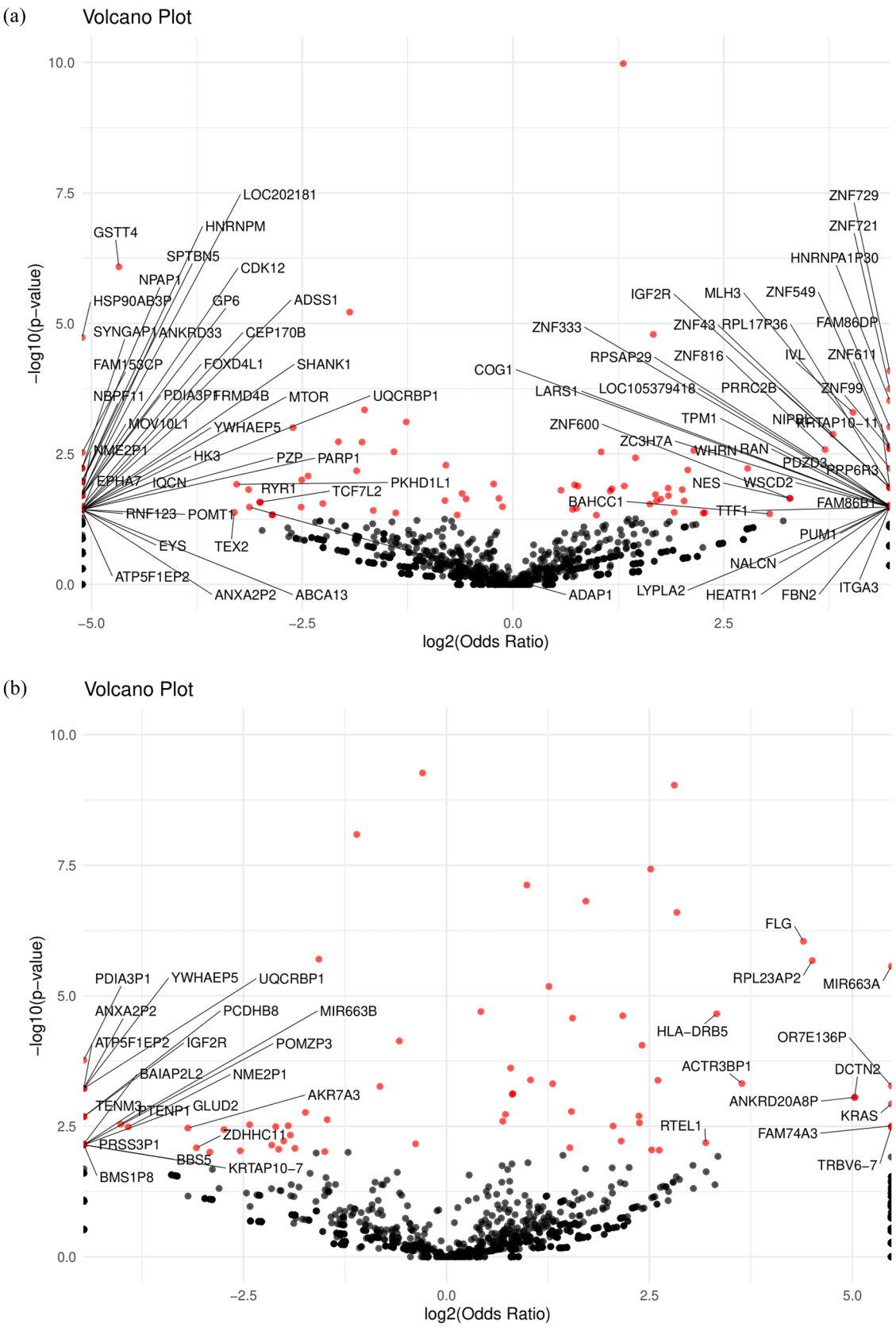

Fig. 2. ctDNA WES identifies fast progressing patients and liver tropism. A. Volcano plot illustrating the proportion of somatic mutations in the baseline plasma of patients with survival under 11 months (negative Odds ratio values) versus those with survival greater than 11 months (positive Odds ratio values). B. Volcano plot depicting the proportion of somatic mutations in the baseline plasma of patients with metastatic tropism to sites other than the liver (negative odds ratio values) versus those with liver-specific metastatic tropism (positive odds ratio values). The red dots indicate Fisher test p-values less than 0.05.

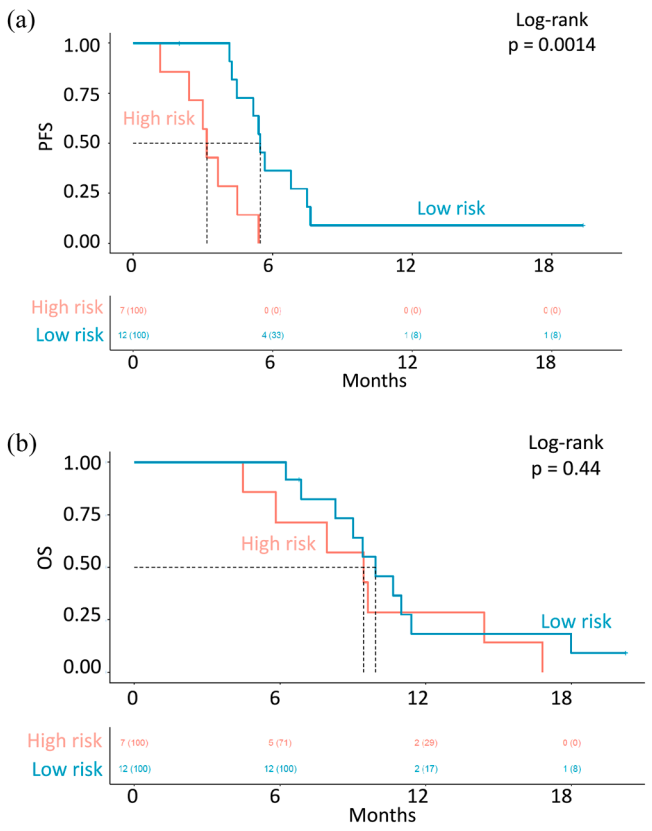

Fig. 3. Kaplan-Meier estimates of progression-free survival (A) and overall survival (B) in advanced pancreatic cancer patients. Kaplan-Meier survival curves comparing patients in the validation cohort with advanced pancreatic cancer. The high-risk group consists of patients with a ≤84.74 % reduction in the VAF of *KRAS* in plasma, while the low-risk group includes those with a >84.75 % reduction.

assessment was associated with worse OS (HR= 2.923, CI 95 %= 1.119–7.636, $p = 0.022$), whereas detecting *KRAS* mutations at baseline had no impact on OS (p= 0.074). These findings were confirmed in a validation cohort of 43 patients (Supplementary Fig. S5 C–D).

Analysis of both discovery and validation cohorts revealed that patients consistently positive for *KRAS* mutations in both baseline and response assessments had inferior PFS compared to those consistently negative ($p = 0.003$), and also experienced worse PFS than individuals transitioning from positive to negative status ($p = 0.003$) [Supplementary Fig. S6].

An optimal threshold for *KRAS* VAF reduction was identified to differentiate prognoses in the discovery and validation cohorts. A minimum 84.75 % decrease at initial response assessment compared to baseline correlated significantly with differences in PFS (Discovery cohort, $p = 0.006$; Validation cohort, $p = 0.001$, Fig. 3). Multivariable Cox regression analysis, optimizing the model based on Akaike's Information Criterion, revealed ECOG status and *KRAS* VAF reduction in plasma to be the most influential predictors. The model with the lowest Akaike's Information Criterion (59.77) showed *KRAS* VAF reduction as the sole significant predictor (HR = 6.71, 95 % CI = 1.77–25.38, $p = 0.005$, Table 3). Conversely, CA19.9, a standard blood-based biomarker for monitoring PDAC treatment response, did not emerge as a prognostic marker in this analysis (HR= 3.37, 95 % CI= 0.68-16.83, p= 0.139).

Discussion

This study is the first to compare the presence of somatic mutations in WES of PDAC plasma samples versus tissue or panel sequencing. We conducted a comprehensive study using WES within a complex cohort of mPDAC,[39] in which we profiled molecular characteristics in plasma, WBCs, and TT at baseline and assessed plasma samples through ddPCR after initial tumor assessment.

Plasma analysis provided a less invasive approach to identify molecular alterations beyond the primary tumor in the cohort analyzed, underscoring the utility of ctDNA in PDAC compared to other tumors.[14,40,41] We identified mutations in well-established PDAC genes (*KRAS, TP53, CDKN2A*, and *SMAD4*) in both plasma and TT, with proportions differing slightly from reported data[8,42-44] and revealing more frequent alterations in plasma than TT. This highlights the added information yielded from plasma analysis, which unveiled a greater aggregate of mutations, including targetable modifications not detected in tissue-based analyses (Table 2).

Unlike prior studies, in which next generation sequencing analyses were limited to a specific gene set,[45] we made an extensive exploration of detectable mutations using WES. Initially, we focused on a PDAC hallmark-associated panel of 30 genes for analysis and pathogenic variant detection.[35] Via WES we were able to uncover additional alterations from databases like COSMIC (v94), OncoKB API (v1), and our in-house pathogenic mutation database. The discovery of novel oncogenic mutations could delineate subtypes in pancreatic cancer with unique attributes and response to therapies.[36] This data holds promise for innovative therapeutic strategies and personalized medicine. Importantly, by limiting the analysis to tissue-based WES alone, some of these alterations might have been missed.

In addition to somatic alterations, WES analysis of WBCs detected germline pathogenic alterations in our cohort, revealing *TP53, RECQL4*, and *AXIN1* mutations (Suppl. Table S1). One patient with a *TP53* germline mutation had Li-Fraumeni syndrome, which elevates the risk of PDAC and other tumors.[46] Other germline mutations, such as *RECQL4* impacting chromosome segregation or *AXIN1* regulating the WNT1 pathway,[47,48] are associated with cancer susceptibility. WBC analysis distinguishes somatic from germline alterations, identifying high-risk patients and enabling early diagnosis through genetic counseling.[49]

Table 3
Univariable and multivariable Cox regression in the validation cohort, displaying the hazard ratio (HR) and confidence interval (CI) for each analyzed variable.

		Univariable		Multivariable	
		HR (CI 95 %)	p-value	HR (CI 95 %)	p-value
Age	**< 65 years**	-	-	-	-
	≥65 years	0.63 (0.23-1.79)	0.390	-	-
ECOG status	**0**	-	-	-	-
	1	1.94 (0.24-15.51)	0.532	1.48 (0.18-12.53)	0.718
	≥2	12.93 (0.99-169.09)	0.051	9.31 (0.60-143.64)	0.110
Liver metastasis	**No**	-	-	-	-
	Yes	1.01 (0.27-3.69)	0.994	-	-
VAF reduction biomarker	**Low risk**	-	-	-	-
	High risk	7.12 (1.97-25.77)	0.003	6.71 (1.77-25.38)	0.005
CA 19.9 level	**High**	-	-	-	-
	Low	3.37 (0.68-16.83)	0.139	-	-

In this study we identified two progression profiles, defined as fast and slow progressing patients. Those with a poorer prognosis in standard therapy harbor mutations exclusively detected in baseline plasma, including prominent oncogenes like *MTOR*, *CDK12*, and *EPHA7* (Fig. 2A). Interestingly, *CDK12* has also been associated with a dismal prognosis in other solid tumors such as prostate cancer, and the development of therapeutic agents targeting this mutation is ongoing.[50,51]

Colonization of distant organs is the most clinically dramatic and biologically complex phase of the metastatic process.[52] In our study, 68 % of patients presented with liver metastases at diagnosis, confirming a propensity for liver tropism in PDAC. Consistent with other studies,[53] patients developing only liver metastases in our cohort exhibited somatic mutations in *KRAS*. Interestingly, these patients also had mutations in genes involved in the adaptive immune response pathway (Fig. 2B), suggesting the role of tumor microenvironment and immune system in their development. It could therefore be liver tropism, rather than the presence of peritoneal lesions, that is mediated by mechanisms involving immune evasion and mutations in proliferation pathways. Further studies are warranted to validate this theory. ctDNA monitoring can detect treatment-induced changes and correlate them with response.[17,54,55] ddPCR was used to assess *KRAS* mutations at diagnosis and during treatment response evaluations in the combined discovery and validation cohort (80 patients) [Suppl. Fig. S1]. Patients with *KRAS* mutations at both time points showed lower PFS in both cohorts (Suppl. Fig. S5). Unlike other series, our analysis found no significant OS differences, likely due to the limited patient sample.[22,28,56] Nevertheless, 25 % (20 out of 80) patients in our cohort had no *KRAS* mutations, making it impractical to monitor treatment response. This study emphasizes the importance of identifying mutations in alternative genes for plasma evaluation in assessing treatment response.

We then assessed *KRAS* detection changes during treatment, finding that patients with undetectable or transitioning to undetectable *KRAS* mutations showed longer PFS than those maintaining detectable mutations (Suppl. Fig. S6). A reduction exceeding 84.75 % in *KRAS* VAF in the discovery and validation cohorts signified improved PFS (Fig. 3). In multivariate analysis with established prognostic factors, this parameter emerged as the sole significant factor. Surprisingly, CA19.9, a routine blood-based biomarker for treatment response, did not serve as a prognostic marker in our cohort.

While the prognostic value of plasma *KRAS* mutations in mPDAC has been established,[22] a novel finding of this study is VAF decrease as a prognostic indicator. The study of ctDNA progression, defined by VAF changes during treatment, can be observed in other tumors[30] and is included in various clinical trials for guiding treatment switches and preventing potential drug-related toxicities due to treatment ineffectiveness. Retrospective trials typically assess VAF within four weeks of treatment initiation. Unlike our cohort, where an 84.75 % reduction positively impacted PFS at the two-month mark, some trials use a 30 % VAF reduction for treatment discontinuation. This suggests potential bias in our study due to late VAF evaluation. Integrating ctDNA progression into PDAC trial design can establish the optimal VAF reduction threshold for determining treatment ineffectiveness.

This study explores ctDNA WES for molecular profiling, intratumoral heterogeneity evaluation, and predictive molecular alterations for targeted therapy in mPDAC. The analysis identifies two distinct rates of progression and pathways affecting liver metastasis tropism, providing insights into potential therapeutic strategies. Despite its retrospective design at a single institution, this study represents one of the most extensive ctDNA-based WES series in mPDAC.

In conclusion, our exploratory study establishes the proof of concept for using ctDNA as a minimally invasive tool for comprehensive molecular characterization, identifying targeted treatment options, profiling progression under standard treatment, and determining organ tropism. It also has potential utility as a prognostic biomarker in mPDAC.

Data statement

Data generated during this study are available from the corresponding author upon reasonable request. Code for analyses is available at https://https://github.com/INCLIVA-bioinformatics/INCLIVA-pancreatic-cancer. The datasets used to generate the figures in the current study are also accessible in the repository.

CRediT authorship contribution statement

Marisol Huerta: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Jorge Martín-Arana:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francisco Gimeno-Valiente:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Juan Antonio Carbonell-Asins:** Formal analysis, Data curation. **Blanca García-Micó:** Writing – original draft, Data curation, Conceptualization. **Belén Martínez-Castedo:** Data curation. **Fabián Robledo-Yagüe:** Formal analysis, Data curation, Conceptualization. **Daniel G. Camblor:** Data curation. **Tania Fleitas:** Resources, Conceptualization. **Miguel García Bartolomé:** Resources. **Clara Alfaro-Cervelló:** Validation, Resources. **Marina Garcés-Albir:** Methodology, Conceptualization. **Dimitri Dorcaratto:** Resources. **Elena Muñoz-Forner:** Conceptualization, Data curation, Formal analysis. **Víctor Seguí:** Investigation, Formal analysis. **Isabel Mora-Oliver:** Methodology. **Valentina Gambardella:** Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Susana Roselló:** Resources. **Luis Sabater:** Writing – original draft, Resources, Conceptualization. **Desamparados Roda:** Resources, Conceptualization. **Andrés Cervantes:** Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Noelia Tarazona:** Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

AC declares institutional research funding from Genentech, Merck Serono, BMS, MSD, Roche, Beigene, Bayer, Servier, Lilly, Natera, Novartis, Takeda, Astellas and Fibrogen and advisory board or speaker fees from Merck Serono, Roche, Servier, Takeda and Astellas. NT declares advisory board or speaker fees from Merck Serono, Servier, Pfizer, Natera and Guardant Health. MH declares advisory board and speaker fees from Servier. TF declares institutional research funding from Genentech, Adapt immune, Roche, Beigene, Astelas, BMS, Daichii Sanyo, Amgen and speaker fees from Astrazeneca, Amgen, Bayer, BMS, Lilly, MSD and Servier. VG declares advisory board fees from Boehringer Ingelheim and institutional research funding from Bayer, Boehringer, Roche, Genentech, Merck Serono, Beigene, Servier, Lilly, Novartis, Takeda, Astelas, Fibrogen, Amcure, Natera, Sierra Oncology, AstraZeneca, Medimmune, BMS, MSD. SR declares personal fees as an invited speaker from Amgen, MSD and Servier. Advisory board fees from Amgen, Servier and Sirtex and institutional funding from Ability Pharmaceuticals, Astellas, G1 Therapeutics, Hutchinson, Menarini, Mirati, Novartis, Pfizer, Pierre Fabre, Roche and Seagen. LS declares advisory board or speaker fees from Johnson and Johnson, Medtronic, Baxter, Cella Medical Solutions. The remaining authors declare no competing interests. All authors have read the journal's policy on disclosure of potential conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.trsl.2024.05.003.

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